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Review Article

Epigenetic targets as an approach to cancer therapy and chemoprevention

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Abstract

Carcinogenesis and tumor progression are controlled by both genetic and epigenetic events. Unlike genetic aberrations, epigenetic aberrations such as DNA methylation and histone deacetylation evident in cancer cells can usually be reversed to reactivate epigenetically silenced tumor suppressor genes and possibly normalize a malignant cell population. Thus, researchers have focused on epigenetic events as targets for effective cancer therapy and chemoprevention. At the 97th American Association for Cancer Research Annual Meeting in Washington, D.C., the preclinical and clinical activity of several histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors was described, indicating promising anticancer effects.

Introduction

Carcinogenesis and tumor progression are controlled by both genetic and epigenetic events. Epigenetic phenomena, evident in all biological processes, involve mitotic and meiotic heritable states of gene expression that are not due to alterations in DNA sequences. Unlike genetic changes where reversal is difficult or impossible, epigenetic aberrations can be reversed to reactivate epigenetically silenced tumor suppressor genes and possibly normalize a malignant cell population (Fig. 1). Researchers have therefore focused on epigenetic events as targets for effective cancer therapy and chemoprevention (1-5).

Several enzyme families are involved in epigenetic events. These include DNA methyltransferases (DNMTs), histone acetylases (HATs), histone deacetylases (HDACs), histone lysine methyltransferases (HMTs) and histone demethylases. All these enzymes can interact directly with DNA or histone tails, introducing modifications and thus changes in gene expression. DNA methylation and histone modification are the two epigenetic events that together intricately control the status of gene expression and ultimately determine the fate of a cell. Because human tumors commonly exhibit changes in DNA methylation and histone modifications, researchers have focused on the identification of epigenetic agents such as HDAC and DNMT inhibitors as potential anticancer agents. Inhibition of DNMTs and/or HDACs could result in inhibition or reversal of epigenetic silencing in cancer cells. The involvement of these two enzymes in epigenetic events has been extensively studied, resulting in the identification of several HDAC and DNMT inhibitors with antitumor effects that have progressed to clinical testing, as shown in Tables I and II (3-15).

Several studies presented at the 97th American Association for Cancer Research (AACR) Annual Meeting in Washington, D.C. presented novel data on DNMT and HDAC inhibitors.

Both preclinical and early clinical activity was reported, indicating promising antitumor effects for epigenetic targeting agents.

Histone deacetylase (HDAC) inhibitors

Histone is a highly conserved protein found in the nuclei of all eukaryotic cells, where it is complexed to DNA in chromatin and chromosomes. It is a protein of relatively low molecular weight and is basic, with a high arginine/lysine content. Two copies of histones H2A, H2B, H3 and H4 bind to about 200 base pairs of DNA to form the repeating structure of chromatin known as the nucleosome; H1 binds to the linker sequence. Histone can act as a nonspecific repressor of gene transcription, and histone acetylation in particular is one mechanism which regulates chromatin structure and its transcription. HDAC is the enzyme that removes an acetyl group from histones, allowing them to bind DNA and inhibit gene transcription. Inhibitors of HDAC can transcriptionally reactivate dormant tumor suppressor genes. In addition, these agents exhibit cell cycle-arresting and proapoptotic properties and induce chromatin remodeling and loss of fidelity during mitosis, although the exact mechanism of these actions is unknown (Fig. 1). Inhibitors of HDAC can be divided into four groups: short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides and benzamides (Table I) (16-22).

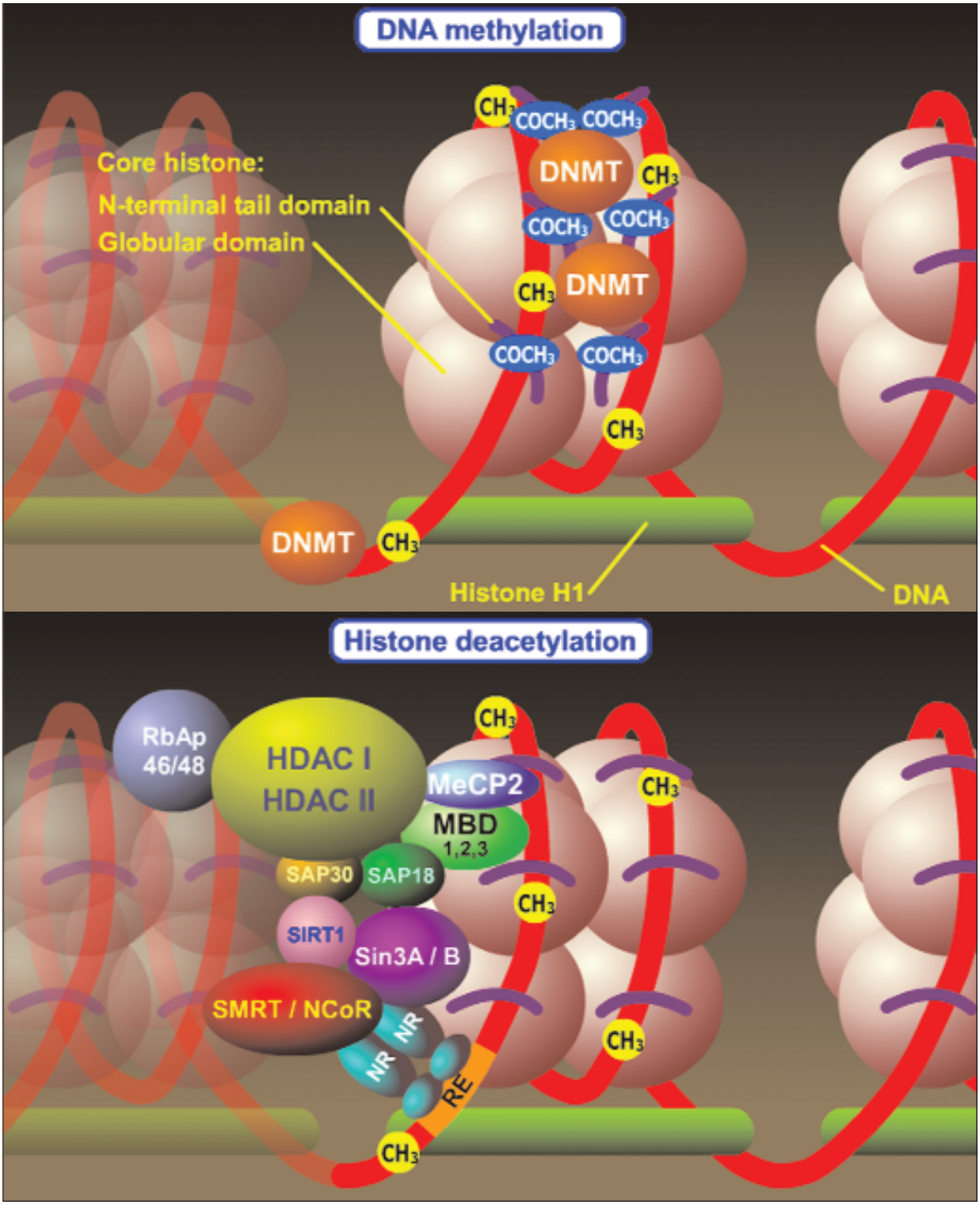
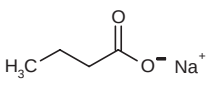


Fig. 1. A nucleosome octamer contains two copies of core histone proteins, H23A, H2B, H3 and H4, each of which has two distinct domains: the globular histone-fold domain and the lysine-rich, positively charged, *N*-terminal tail. Histone H1 fastens the DNA to the nucleosome core and helps pack nucleosomes together in the fiber. The silencing process of a gene begins with the recruitment of DNA methyltransferases (DNMTs) resulting in DNA methylation. After methylation, methyl-CpG-binding protein 2 (MeCP2) and methyl binding domain (MBD) proteins 1, 2 and 3 are recruited, which will recruit more silencing factors. In the absence of ligands, nuclear receptor dimers are associated with co-repressor complexes (SMRT/NCoR) that recruit histone deactylases (HDAC) either directly or indirectly through their interaction with enzymatic complexes. Deacetylation of the histone tail leads to chromatin compaction and transcriptional repression.

Table I: Histone deacetylase (HDAC) inhibitors under development for the treatment of cancer.

Inhibitor	Source	Phase
Short-chain fatty acids		
1. Sodium butyrate (217909)	INSERM/Osaka Medical Center for Cancer & Cardiovascular Diseases	I
2. Valproic acid (280482)	Abbott	I
Hydroxamic acids		
3. Vorinostat (230535)	Merck & Co.	II/III
4. PXD-101 (318295)	CuraGen/TopoTarget	II
5. LBH-589[1] (353509)	Novartis	I
6. Pyroxamide (287630)	National Cancer Institute (US)	I
7. Oxamflatin (239622)	Shionogi/TopoTarget	Preclinical
8. CBHA (303699)	Cornell University	Preclinical
9. Trichostatin A (115620)	Ajinomoto	Preclinical
10. Scriptaid (314106)	Johns Hopkins University	Preclinical
11. HDAC-42 (383147)	Indiana University/Ohio State University	Preclinical
12. (*S*)-HDAC-42 (403161)	Ohio State University	Preclinical
13. SB-623 (423355)	S*BIO	Preclinical
14. SB-624 (401579)	S*BIO	Preclinical
15. SB-639 (423356)	S*BIO	Preclinical
16. SK-7041 (355164)	In2Gen/SK Chemicals	Preclinical
17. CRA-026440[1,2]	Celera Genomics	I



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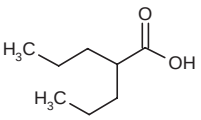

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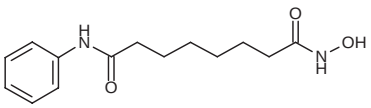

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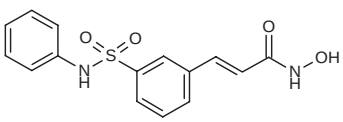

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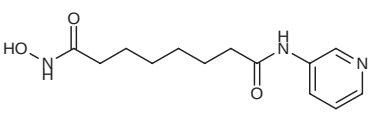

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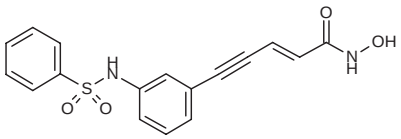

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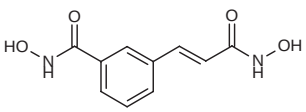

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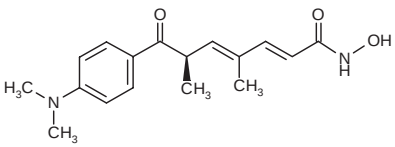

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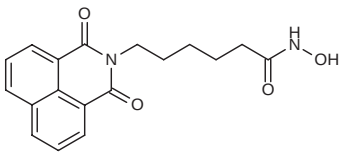

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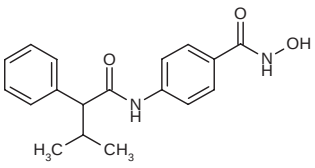

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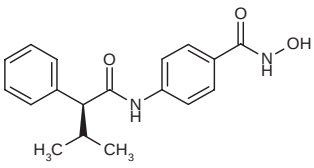

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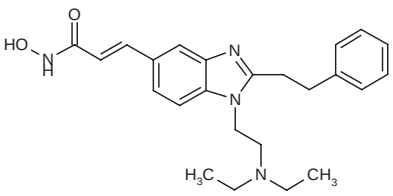

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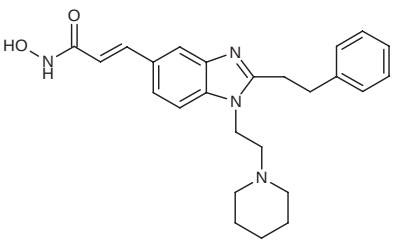

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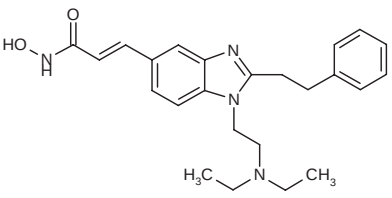

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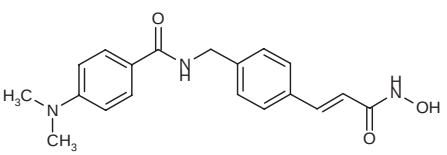
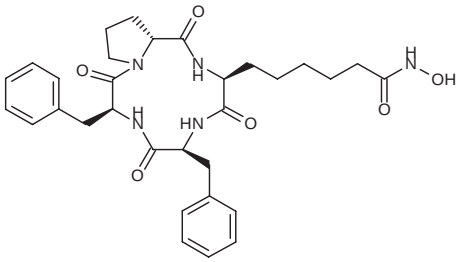


(**16**)

Continuation

Table I (cont.): Histone deacetylase (HDAC) inhibitors under development for the treatment of cancer.

Inhibitor	Source	Phase
Cyclic tetrapeptides		
18. Romidepsin (158963)	Astellas Pharma/Gloucester Pharmaceuticals/National Cancer Institute (US)	III
19. Apicidin (235761)	Merck & Co.	Preclinical
20. CHAP-1 (125751)	Japan Energy	Preclinical
21. Trapoxin B (171115)	Shionogi	Preclinical
Benzamides		
22. MS-27-275 (266686)	National Cancer Institute (US)/Schering AG	II
23. Compound (**23**) (423965)	MethylGene	Preclinical



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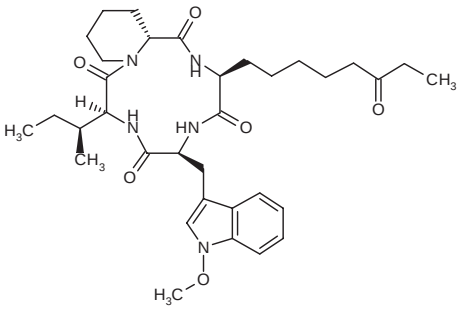

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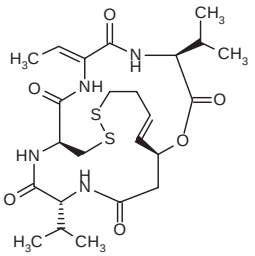

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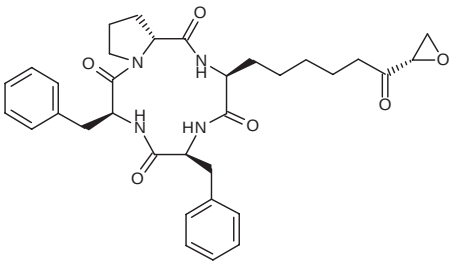

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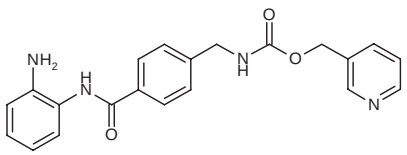

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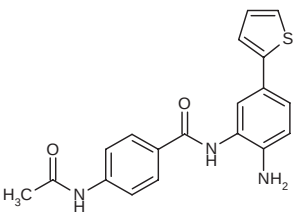


(**23**)

[1]Structure not yet available. [2]According to Prous Science Integrity®, the company is studying hydroxamic acid derivatives as HDAC inhibitors (WO 2003066579; WO 2004092115; WO 2005019174; WO 2005097770).

Several pan-HDAC inhibitors have progressed to clinical trials, with results showing good tolerability and suggesting that, when administered in association with classic chemotherapeutic drugs or in combination with DNA-demethylating agents, these agents could be promising drugs for cancer patients. Highlights of some of the preclinical and clinical studies presented at the AACR meeting are summarized below.

Researchers from MethylGene have identified a novel class of thiophenyl-substituted benzamides that exhibit HDAC-inhibitory activity, enhanced isotype selectivity and antiproliferative actions *in vitro* and *in vivo*. Compound (**23**) is a thiophenyl derivative of tacedinaline (CI-994) that was shown to have significantly enhanced HDAC-inhibitory activity in enzyme experiments. Antiproliferative activity was also observed in human cancer cell lines and the compound was effective *in vivo* in murine xenograft models (23).

Novel *N*-hydroxy-3-benzimidazol-5-ylacrylamides were described by researchers at S*BIO as prototypes for HDAC inhibitors. The agents were more potent then suberoylanilide hydroxamic acid (vorinostat, SAHA) or PXD-101 *in vivo*, SB-623, SB-624 and SB-639 emerging as the most promising agents of this class (IC_{50} for HDAC1 = 51, 24 and 35 nM, respectively). The agents effectively inhibited the proliferation of COLO 205, MDA-MB-231, PC-3 and A549 tumor cell lines *in vitro,* with GI_{50} values ranging from 87 to 320 nM. All compounds were stable in human liver microsomes ($t_{1/2}$ > 30 min) and showed no interaction with cytochrome P-450 (CYP) 2D6 or 3A4 enzymes. The agents were well tolerated when given i.p. for 21 days to mice, with a maximum tolerated dose (MTD) of > 50 mg/kg i.p. SB-639 (100 or 130 mg/kg/day i.p. or 100 or 200 mg/kg/day p.o. for 21 days) exerted potent tumor growth-inhibitory activity in nude mice bearing HCT 116 xenografts. After 21 days of treatment, tumor growth-

inhibitory rates were 52%, 81%, 44% and 70% for the respective doses, and significant tumor growth delays were observed as compared to controls (24).

The novel, broad-spectrum HDAC inhibitor CRA-026440 (Celera Genomics) was shown to have potent inhibitory activity against HDAC enzymes (K_i = 4-20 nM) and growth-inhibitory effects against several human cancer cell lines *in vitro*. The agent was also effective *in vivo* when administered in murine xenograft models as monotherapy or in combination with bevacizumab (Avastin®) or 5-fluorouracil (5-FU). CRA-026440 significantly reduced the growth of U-937 histiocytic lymphoma and HCT 116 xenografts. However, CWR-22RV1 prostate tumor xenografts were resistant to treatment. Examination of the mechanism of action revealed that the agent downregulates the tumor angiogenesis-related genes VEGF and KDR. In addition, CRA-026440 was shown to time-dependently induce Erk and p70 (S6) phosphorylation and increase STAT3 levels and caspase-3 activation in HCT 116 cells (25).

The preclinical activity of the novel, rationally designed phenylbutyrate HDAC inhibitor HDAC-42 was reported by researchers from Indiana University and Ohio State University. HDAC-42 was developed to specifically insert into the active-site pocket of HDAC and was shown to exert both classic HDAC inhibitor effects, such as acetylating H3, and nonhistone effects, such as reducing α-tubulin deacetylation. The antiproliferative effects of the agent were demonstrated in experiments using several ovarian cancer cell lines. IC_{50} values obtained against SK-OV-3, A2780 and cisplatin-resistant CP70 cells following a 72-h treatment were 1.9, 0.4 and 0.6 μM, respectively. HDAC-42 was less toxic than vorinostat in experiments using normal ovarian surface epithelial cells (IC_{50} = 8.9 μM *vs*. 6.3 μM). Analysis of the mechanism of action of HDAC-42 revealed that the agent acts via both p53-dependent and -independent cascades, since it upregulated *NOXO* 3-fold and gammaglobin 45-fold in the p53-positive cell line A2780. However, *p21*, the p53 target gene, was upregulated in both p53-negative and -positive cells, suggesting that p53 is not fully needed for induction (26).

The effects of HDAC-42 on ErbB family receptors were investigated in T24 (high-grade invasive transitional cell carcinoma) and RT4 (low-grade noninvasive papillary transitional cell carcinoma) bladder cancer cell lines. Treatment with 25-100 nM resulted in significant, concentration-dependent increases in H3 and H4, indicating HDAC inhibition. Cell viability and the expression of ErbB receptors were not altered by treatment even up to 96 h. However, treatment for 24 h with 1-5 μM resulted in significant and concentration-dependent decreases in ErbB-1 and ErbB-2 expression in both cell types. Inhibition of receptor expression was found to be occurring at the transcriptional level and was associated with reduced phosphorylated ERK1/2 levels. This suggests that the agent acts downstream in the Raf-1/MEK/ERK pathway. HDAC-42 was also shown to significantly decrease ErbB-4 expression in RT4 but not T24 cells, and to induce expression of ErbB-3 in T24 cells. The results suggest that HDAC-42 may be effective in combination with anti-Erb-B agents (27).

To evaluate the potential efficacy of HDAC-42 as a treatment for esophageal adenocarcinoma (EAC), its effects on acid-induced gene expression patterns of acid-pulsing SEG-1 human EAC cells were examined. Treatment of the SEG-1 cells with the agent caused an overrepresentation of 10 gene categories, of which the five most significantly upregulated categories included cellular process, cell communication, endosome, protein binding and signal transduction. In addition, among the significantly downregulated genes, 113 categories were significantly overrepresented, with mitotic cell cycle, cell cycle, M phase, nucleus, nuclear division and cell proliferation the most significantly downregulated categories. Both acid-pulsing SEG-1 cells and cells pretreated with HDAC-42 followed by acid pulsing did not exhibit significantly overrepresented gene categories, suggesting that acid treatment does not target specific gene categories. However, many probe sets were significantly up- or down-regulated in HDAC-1-pretreated acid-pulsing cells (28).

Results from another *in vitro* study suggest that (*S*)-HDAC-42 may be effective as a radiosensitizer for the treatment of prostate cancer. The study examined the antitumor effects of the agent in combination with ionizing radiation (2 Gy) against PC-3, DU 145 and LN-3 prostate cell lines. The LN-3 cell line was the most sensitive to (*S*)-HDAC-42. Treatment with the agent significantly blocked cells in the G2/M phase of the cell cycle and increased Bax and NF-κB levels while decreasing Bcl_{XL} and Bcl-2 levels. In addition, nuclear p65 decreased with treatment, suggesting that nonepigenetic mechanisms, such as alterations in nucleo-cytoplasmic shuttling, may also be involved in the antineoplastic effects seen with combination treatment (29).

The efficacy of SK-7041, an inhibitor of class I HDACs from SK Chemicals, as a radiosensitizer in human cancer cells was examined and compared to the HDAC class III inhibitor splitomicin and the class I and II inhibitor TSA. TSA was the most effective radiosensitizer, followed by SK-7041 and splitomicin, which was the least potent. The radiosensitizing effects of TSA were more marked in cells expressing p53 (30).

The novel hydroxamate HDAC inhibitor PXD-101 (CuraGen; TopoTarget) was shown in *in vitro* studies to be effective as monotherapy (at submicromolar to low micromolar concentrations) and in combination with the EGFR inhibitor erlotinib against several human non-small cell lung cancer (NSCLC) cell lines. Combination treatment resulted in additive to synergistic effects and PXD-101 treatment alone was shown to reduce EGFR expression (31).

An *in vitro* study using five human melanoma cell lines demonstrated that full-length TNF-related apoptosis-inducing ligand (TRAIL) delivered by an adenoviral vector (Ad-hTRAIL) combined with the HDAC inhibitor vorinostat decreased cell growth at 72 h posttreatment in four cell lines; the growth-inhibitory effects observed were additive or synergistic. However, combination treatment including

Ad-hTRAIL and dacarbazine was more effective in inhibiting cell growth than Ad-hTRAIL and vorinostat (32).

Vorinostat is currently undergoing clinical development for the treatment of various cancers. A phase I study in 23 patients with advanced cancer examined the safety, tolerability and pharmacokinetics of the agent (400 mg/day for 28 days) in the fasted and fed states. Vorinostat was well tolerated. Adverse events related to treatment included thrombocytopenia, increased serum creatinine, anorexia/weight loss, nausea/vomiting and fatigue. Twenty-three and 14 patients were evaluable for day 1 single-dose and day 28 multiple-dose pharmacokinetics, respectively. The rate of absorption of the agent was reduced in the presence of a standard high-fat meal. In the fed state, most patients had a delay of a minimum of 15 min before levels of vorinostat could be detected in serum and the t_{max} was slightly delayed. Mean apparent terminal $t_{1/2}$ values were short and similar for both single and multiple doses and for the fed and fasted states (1.36-1.74 h) (33).

An *in vivo* study using BALB/c mice bearing renal cell carcinoma (RENCA) xenografts showed that combination treatment including the HDAC inhibitor MS-27-275 (5 mg/kg p.o. 5 days/week for 2 weeks) and interleukin-2 (IL-2; 150,000 IU i.p. b.i.d. twice weekly for 2 weeks) resulted in synergistic activity. Treatment was initiated 4 days postimplantation and significant reductions in tumor growth of 80% or more were observed with combination treatment as compared to IL-2 alone, which produced no significant effects, and MS-27-275 alone, which caused an approximate 40% inhibition. Significant reductions in spontaneous lung metastases (> 90%), greater splenocytic lysis, increased lymph node $CD4^+$ cells, reduced lymph node Fox-$p3^+$ cells and a significant increase in survival were also observed with combination treatment as compared to either monotherapy (34).

LBH-589 was shown to reactivate the silenced estrogen receptor α (ERα) gene in ER-negative human breast cancer cell lines (MDA-MB-231, MDA-MB-435). Treatment with the HDAC inhibitor for 24 h restored ER mRNA and protein expression, effects that were sustained for at least 96 h after drug withdrawal. The mechanism by which it restored the silenced ER gene did not involve demethylation of the CpG island at the ER promoter. However, LBH-589 caused release of DNMT1, HDAC1 and the H3 (Lys-9) methyltransferase SUV39H1 from the ER promoter, which was associated with accumulation of acetylated histones H3 and H4 (indicating euchromatin formation), a decrease in DNMT1 protein expression, a decrease in H3 (Lys-9) methylation, an impairment of heterochromatin protein 1 (HP1) binding at the promoter, and an increase in ER promoter activity (35).

A preclinical study evaluated the efficacy of combination treatment targeting hypoxia-inducible factor-1α (HIF-1α) and tissue factor (TF) by the mTOR inhibitor rapamycin combined with the HDAC inhibitor LBH-589. Treatment of endothelial cells and a renal cell carcinoma cell line (UMRC2) deficient in Von Hippel-Lindau tumor suppressor gene (VHL) resulted in a significant decrease in HIF-1α expression as compared to either monotherapy. In addition, LBH-589 completely blocked rapamycin-induced TF activity in VEGF-stimulated human umbilical vein endothelial cells (HUVEC), and TF expression in UMRC2 cells was significantly inhibited by about 50%. Treatment with the combination in an orthotopic UMRC2 model resulted in significant antitumor and antiangiogenic effects. No rapamycin-induced intratumoral thrombosis or increase in TF expression was observed. Results suggest that LBH-589 prevents VEGF- and rapamycin-induced TF activity (36).

LBH-589 (20 nM) was also shown to act synergistically with the multiple receptor kinase inhibitor AEE-788 (5 μM) to induce apoptosis in lung cancer cells (A549). Each agent alone caused little cytotoxic effect, while combination treatment markedly increased cytochrome *c* release, caspase activation, PARP (poly[ADP-ribose]polymerase, NAD^+ ADP-ribosyltransferase) cleavage and apoptosis. In addition, Mcl-1, a member of the Bcl-2 antiapoptotic family, phospho-p70S6K and p21 were downregulated in cells treated with both agents. Similar effects were also observed when AEE-788 was combined with other HDAC inhibitors, such as LAQ-824 or sodium butyrate (37).

LBH-589 is currently undergoing phase I development as both i.v. and oral formulations, and preliminary results were reported from an ongoing phase I trial in 9 patients with advanced-stage cutaneous T-cell lymphoma (CTCL). Patients were treated with either 20 or 30 mg 3 times per week until disease progression or unacceptable toxicity. Complete and partial responses were obtained in 2 patients each and 1 patient had stable disease with ongoing improvement; 4 patients had disease progression. Two patients who had stable disease discontinued due to grade 3 diarrhea at week 4 and grade 2 fatigue at week 12. However, 3 months later, complete and partial responses, respectively, were achieved by these patients, indicating ongoing disease regression even after treatment discontinuation. A patient who responded with a complete response was discontinued after 10 doses due to grade 3 diarrhea and progressed at 8 months. Distinct gene expression response profiles were obtained for 3 patients with a complete response and 1 with disease progression (38).

Other HDAC inhibitors under development are also shown in Table I.

DNA methyltransferase (DNMT) inhibitors

DNA is methylated by DNMTs at the 5-position of the cytosine ring (C5), almost exclusively at CpG dinucleotides. Deviations in the expected methylation patterns usually established during development and the associated changes in gene expression can lead to tumorigenesis. Cancer cells exhibit changes in 5-methylcytosine distribution, such as global DNA hypomethylation and hypermethylation of promoter CpG islands associated with tumor suppressor genes. CpG island hypermethyla-

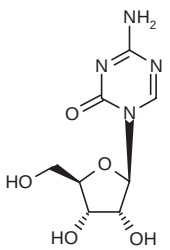
tion represents a change in the chromatin structure that together with histone modifications can result in a transcriptionally silenced state (see Fig. 1). DNA methylation is an excellent marker for assessing the epigenetic state of a locus due to the fact that it is preserved in isolated DNA and it has been used to globally profile cancers. DNMTs are responsible for both *de novo* methylation and its maintenance, and DNMT inhibition could therefore restore DNA methylation patterns. Only three of the five DNMTs identified to date possess enzymatic activity (DNMT1, DNMT3a and DNMT3b *vs*. DNMT2 and DNMT3L). There are currently two recognized classes of DNMT inhibitors: nucleoside analogues and non-nucleoside analogues (Table II). Nucleoside analogues have a modified cytosine ring attached to either a ribose or deoxyribose moiety. These agents are metabolized by kinases, which convert nucleosides to nucleotides, and are then incorporated into DNA (and RNA) where they inhibit DNA methylation. Non-nucleoside analogues may be more effective since they are small-molecule inhibitors that bind directly to the catalytic region of DNMT, rendering the enzyme inactive; they are not incorporated into DNA (39-42). Several studies presented at this year's AACR meeting indicated potential efficacy for selected DNMT inhibitors as cancer therapeutics.

The nucleoside DNMT inhibitor zebularine is a synthetic ribonucleoside that is thought to exert antineoplastic effects through reactivation of tumor suppressor genes that are dormant due to the aberrant DNA methylation occurring in cancer cells. The agent is stable in both aqueous and acid solutions, is cancer cell-selective and less toxic than 5-azacytidine and decitabine. Zebularine was also shown to be effective in a murine T-cell lymphoma xenograft model. The safety and tolerability of the agent were examined in mice and cynomolgus monkeys. The effects of long-term administration of zebularine (0.1 mg/kg p.o. for 93 days starting on day 7 after birth) in wild-type and *Apc*$^{min/+}$ mice (genetically predisposed to intestinal and mammary tumorigenesis) were examined. No toxicities or abnormalities were observed in any of the mice throughout the treatment period. Analysis of treated *Apc*$^{min/+}$ mice showed that the agent did not detrimentally alter the gene profile of normal liver tissue. Analysis of DNA methylation in heart, liver, spleen and large and small intestines revealed that the agent was tissue-selective, with a decrease in activity observed only in the large intestine in normal mice (43).

The safety of biologically active doses of zebularine as a continuous infusion and as repeated i.v. boluses for up to two 5-day cycles was examined in male cynomol-

Table II: DNA methyltransferase (DNMT) inhibitors under development for the treatment of cancer.

Inhibitor	Source	Phase
1. Azacitidine* (91171)	Pfizer	L-2004 (myelodysplasia) I
2. Decitabine* (125366)	MGI Pharma	Prereg. (myelodysplasia) III
3. DHAC* (90632)	Pharmachemie	II
4. FdCyd* (287220)	Beckman Research Institute, City of Hope	I
5. Zebularine* (330024)	Univerisity of North Carolina, Chapel Hill	Preclinical
6. Hydralazine hydrochloride (91486)	Novartis	I
7. Psammaplin A (285465)	Korea Research Institute of Chemical Technology	Preclinical
8. RG-108 (406543)	Institute of Biochemistry & Biophysics PAS	Preclinical



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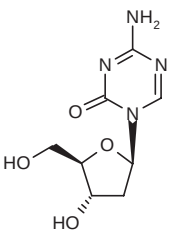

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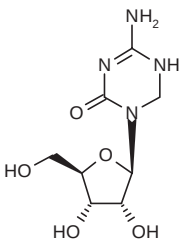

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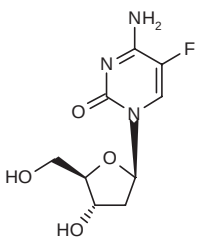

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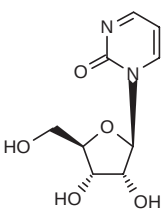

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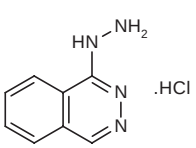

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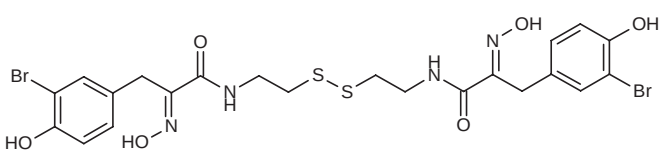

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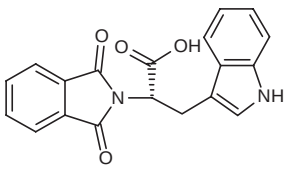

(**8**)

*Nucleoside analogue.

gus monkeys, with results indicating a narrow dose range separating minimal toxicity and unpredictable mortality, possibly due to renal and/or hepatic toxicity-induced impairment in drug clearance and consequent increases in plasma drug levels. Continuous infusion of doses to achieve plasma levels of 10-15 μM resulted in dose-related toxicity. No serious toxicity or mortality was observed. Toxicities associated with continuous infusion included alterations in alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and creatine kinase (CK) levels, and increases in white blood cell and reticulocyte counts. However, continuous infusion of doses to achieve plasma levels of 25-50 μM caused mortality. Small increases in the expression of fetal gammaglobin (a marker of zebularine pharmacodynamic activity) were observed with continuous infusion of doses giving plasma levels of 20 μM. Daily i.v. bolus injections of 500, 750 or 1000 mg/kg induced mortality associated with large increases in plasma zebularine levels, probably due to a reduction in clearance. All doses increased gammaglobin levels. Administration of daily bolus doses of 250 mg/kg or less was less toxic and was not associated with mortality. However, clinical alterations suggested modest hepatic and renal toxicity at these doses (44).

Synergistic proapoptotic activity was observed against NSCLC-derived cell lines when the DNMT inhibitor hydralazine (LD_{50} = 50 μM) was combined with a peroxisome proliferator-activated receptor γ (PPARγ) agonist (LD_{50} = 15 μM). Both agents alone significantly and concentration-dependently decreased cell survival. When combined, the agents resulted in an average increase in apoptosis rates from 51% to 105%, with a trend toward G2/M phase cell cycle arrest. Monotherapy and combination therapy induced re-expression of the tumor suppressor gene *p16* and the transcription factors GATA-4 and PAX-5β. Moreover, preliminary results from *in vivo* studies suggested that treatment with either agent alone or in combination was well tolerated, no toxicity being observed. Thus, combination treatment including a DNMT inhibitor and a PPARγ agonist may be an effective approach for preventing progression and inducing regression of lung cancer (45).

The nucleoside analogue decitabine (5-aza-2'-deoxycytidine) was shown in *in vitro* studies using a prostate cancer cell line (DU 145) to increase expression of *GADD45*α, a gene involved in apoptotic pathways that is reduced in this prostate cancer cell line. In addition, decitabine pretreatment increased the cells' sensitivity to docetaxel (46).

The *galectin-3* gene is silenced by methylation in melanoma and an *in vitro* experiment using a murine melanoma cell line (Tm1) showed that treatment with decitabine (10 μM) induced *de novo* expression of the gene. Treatment also resulted in a reduction in cell growth and an increase in sensitivity to cell death. Interestingly, treatment with high concentrations of the HDAC inhibitor trichostatin A (100 and 200 nM) reversed the effects of decitabine on *galectin-3* expression (47).

The cytotoxic mechanism of action of decitabine and azacytidine was investigated *in vitro* using cancer cell lines. Both agents significantly inhibited cell growth and caused G2/M phase arrest. Further analysis of the effects of decitabine revealed that the agent caused marked DNA damage and induced γH2AX, which is known to be induced by double-strand breaks. However, decitabine-induced γH2AX induction was significantly decreased in cell lines deficient in ATM and p53, suggesting that both these signal transduction pathways may be activated in response to decitabine-induced DNA damage (48).

Decitabine (0.2 mg/kg p.o. 5 days/week for 19 weeks) was reported to be effective against carbamate-induced lung tumors in mice. However, no synergistic or additive effects were observed in this model when decitabine was combined with the HDAC inhibitor vorinostat (400 or 1000 mg/kg in the diet for 19 weeks). Lung tumor multiplicity was significantly decreased in animals receiving decitabine (15.3 ± 1.16 *vs.* 23 ± 0.87 in controls) or vorinostat alone (19.5 ± 1.29 and 8.84 ± 0.82, for the respective vorinostat doses *vs*. 26.9 ± 1.42). Combination treatment also resulted in lung tumor reductions (14.5 ± 1.02 and 7.36 ± 0.92, respectively, *vs*. 26.9 ± 1.42), although potency was equivalent to treatment with either agent alone. In contrast to vorinostat which also reduced the average size of tumors and the percentage of carcinogenic tumors, decitabine had no such effects (49).

H1299 lung cancer cells lack *Fhit* and *Wwox* expression due to promoter hypermethylation. Experiments performed *in vitro* have shown that treatment with either the HDAC inhibitor trichostatin A or the DNMT inhibitor decitabine restores *Fhit* and *Wwox* in H1299 cells and renders the cells nontumorigenic when administered s.c. *in vivo*. An *in vivo* study using mice with established H1299 tumors reported that although treatment with decitabine (7.5 mg/kg i.p.) in combination with trichostatin A (700 μg/kg i.p.) had no effect on tumors that were > 200 mm^3, the combination effectively inhibited or reversed growth of tumors that were < 100 mm^3. However, 2 animals died from toxicity associated with combination treatment. The combination regimen was modified so that animals were treated when all tumors were < 100 mm^3 and decitabine and trichostatin A doses were reduced to 5 and 500 mg/kg, respectively. In addition, the agents were administered both by intratumoral and i.p. injections. These alterations were not associated with any deaths and resulted in restoration of *Fhit* and *Wwox* expression, inhibition or regression of tumor growth, decreases in mitotic activity, increases in the apoptotic fraction and caspase-3 activation. In contrast, administration of i.p. trichostatin A had no such effects in this model (50).

The efficacy of combination treatment including decitabine (5 mg/kg i.p.) and the HDAC inhibitor trichostatin A (500 mg/kg i.p.) was examined in immunocompetent B6SF129/1 mice bearing NSCLC tumors (the activated *k-ras* murine adenocarcinoma cell line LKR13). Treatment was initiated 1 week postinoculation and continued weekly for 3 weeks, and resulted in a reduction in mean tumor volume by day 30 (125 mm^3 *vs*. 540 mm^3 in

controls). Moreover, animals inoculated with LKR13 cells pretreated *in vitro* with decitabine and trichostatin A also exhibited a decrease in mean tumor volume (175 mm^3) as compared to controls. All control animals exhibited tumor volumes > 500 mm^3 by day 45, in contrast to none of the decitabine + trichostatin A-treated animals; only 1 animal in the group inoculated with pretreated LKR13 cells had a tumor volume > 500 mm^3 (51).

Decitabine is currently undergoing phase II/III development and promising results from a randomized phase II trial conducted in patients with myelodysplastic syndrome (MDS) were reported. The study, in which decitabine was administered in three regimens (10 mg/m^2/day i.v. for 10 days, 20 mg/m^2/day for 5 days or 10 mg/m^2 s.c. b.i.d. for 5 days), also analyzed the expression status of the tumor suppressor genes $p15^{INK4B}$ and $p21^{Cip/WAF}$ from 54 patients. An overall response rate of 85% was obtained, which included a complete response rate of 33%. The complete response rate for the three groups was 25%, 42% and 29%, respectively. The expression of *p15* was significantly higher in responders as compared to nonresponders at baseline (3.5-fold) and on day 26 (8.9-fold), and increased from baseline with treatment in both populations (3.5- and 1.4-fold for responders and nonresponders, respectively). In contrast, *p21* expression levels were similar at baseline and unchanged following decitabine therapy. Thus, higher *p15* gene expression at baseline was associated with a better clinical outcome with decitabine therapy (52).

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